SEPARATION, IDENTIFICATION, AND QUANTIFICATION OF LOW MOLECULAR WEIGHT NITROGEN CONTAINING COMPOUNDS IN FISH BYPRODUCTS

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SEPARATION, IDENTIFICATION, AND QUANTIFICATION OF LOW MOLECULAR WEIGHT NITROGEN CONTAINING COMPOUNDS IN FISH BYPRODUCTS

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Abstract

There is interest by the fish processing industry in the identification and eventual extraction of higher valued low molecular weight nitrogen compounds from fish byproducts such as stickwater, hydrolysates, fish tissues, and other byproducts. A hydrophilic interaction liquid chromatography (HILIC) method was developed for the separation and quantification of amino acids, small nitrogenous acids and bases, as well as, other nitrogen containing metabolites. The HILIC method developed is a robust and non-derivatizing method for the analyses of aqueous compounds found in freeze dried red salmon whole fish and red salmon byproducts (pre-treated stickwater, post-treated stickwater, and fishmeal). Triplicate samples of all byproducts were obtained from commercial processors in Kodiak, AK. Byproduct samples were extracted and centrifugally filtered through 3000 MW membranes. The identification of low molecular weight compounds in different fractions of fish byproduct showed the partitioning of unbound components during fishmeal processing. Several aqueous nitrogen containing compounds were quantified and comparisons were made of these components in different fish byproduct fractions. This study suggests that creatine, creatinine, taurine, and hypoxanthine are found in elevated concentrations in stickwater and are preferentially partitioned into the stickwater fraction.

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Chapter 1: Introduction

Overview of Fish Byproduct and the Fishmeal and Fish Oil Industry

Fish byproducts are the residual materials left after removal of the primary products such as fillets and roe. In Alaska, the main fish processing byproducts are fish heads, viscera, frames, and shin. Fish byproduct has been used by man for centuries as a livestock feed source and as fertilizers. As processing capabilities advanced, so did the processing of fish byproduct into useful products such as fish silage, fishmeal, fish oil, fish hydrosylates, etc. Today, most nutritional scientists agree that it is the high quality protein, fatty acid composition of fish oils (often rich in omega-3 fatty acids), unique content of minerals, and high concentrations of other metabolites that make fish byproduct ingredients such a nutritionally valuable commodity.

Most commercially processed fish byproduct is converted to fishmeal via a wet reduction process. It is estimated that about 25% of the total world fishery production is processed via wet reduction to fishmeal and other non-food products.[1] The wet reduction of fish byproduct involves grinding and then cooking the byproduct (~95°C). The cooked byproduct is separated with a press or decanter centrifuge into the protein solids fraction called the press cake and a liquid fraction that contains fish oil and an aqueous fraction. This liquid fraction is then separated by centrifugation into fish oil and an aqueous fraction called stickwater. The press cake is then dried to make

fishmeal. The stickwater can be concentrated by evaporation and added back to the wet press cake prior to drying. The oil is usually centrifuged an additional time to remove water and protein particles. Both fishmeal and fish oil are sold on the world market as feed ingredients. The world's largest producer of fishmeals and oil is Peru, and their meal and oil is made from small oily fish harvested for the sole purpose of making meal and oil. By comparison, Alaska produces a small amount of fishmeal and oil, however, these are made only from fish processing byproducts.

The Economic Sustainability of the Fishmeal and Fish Oil Industry

Over the past 20 years (1991-2010), global fishmeal and fish oil production averaged 6.5 and 1.3 million metric tons, respectively.[2] The global production of fishmeal has remained somewhat stagnant over the past decade due to increased protections put in place to stabilize exploited marine fisheries.[3] On the other hand, the growing human population has caused a sharp rise in the need for more aquaculture, and other animal agriculture products to maintain a food supply chain capable of feeding the world. Nearly all of the 6.5 million metric tons of commercially processed fishmeal produced annually has been used in aquaculture, agriculture, and pet foods. It was estimated that in 2002 46% of the commercially processed fishmeal was used in the aquaculture industry, while the poultry, pork, beef, and other industries accounted for 22%, 24%, 1%, and 7% respectively.[4] Many studies

have shown that the incorporation of fishmeal or fish byproduct into feeds produced for dogs, cats, pigs, cows, and poultry leads to an increase in growth and development.[5-9] All of these findings and others have made fishmeal an increasingly valuable commodity in the aquaculture and agriculture industries.

The world aquaculture industry has been growing rapidly, and the use of fishmeal has increased significantly by the aquaculture industry over the past 30 years.[10] Much of the growth in the aquaculture industry is due to increased demand for aquatic food products and the inability to harvest more seafood in a sustainable manner from oceans, lakes, and rivers . World fishmeal and fish oil production is stagnant, because current commercial fishing resources are already being harvested at maximum sustainable levels. There are no more large biomasses of fish left to be exploited for fishmeal and oil production. Overfishing and the resultant restrictions put in place to protect marine fisheries has caused some to question the fishmeal and fish oil industry's economic sustainability.[11] There is also concern in the aquaculture industry, because fishmeal and oil supplies will not increase dramatically in the future while aquaculture production will increase substantially. Most fishmeal and oil is used as preferred ingredients by the aquaculture industry. The issue is that the many carnivorous aquaculture fish species grow more efficiently on diets containing fishmeal and oil than plant

protein meals and oils. Research is being conducted to develop different feeding systems that utilize less fishmeal and oil while maintaining acceptable growth and other economically important characteristics in the production of farmed fish.

The increase in fish production from aquaculture and the stagnant harvests from wild fisheries has the fishmeal and fish oil industry in a quandry, since a substantial part of the production of fish from aquaculture relies on the processing of wild fish or wild fish byproducts to fishmeal and fish oil.[12,13] Currently small pelagic fish caught for whole fish conversion to fishmeal constitute 20 to 30% of annual world commercial fish harvests.[14] Table 1 shows the top eight types of fish landed in US fisheries in 2003 with their respective landings in metric tons.[15]

Table 1: Top eight commercially landed fish in the US.

Type of Fish	Weight landed in 2003 (in metric tons)
Pollock	1,529,000
Menhaden	725,458
Salmon	305,768
Cod	268,428
Flatfish	201,431
Hakes	154,197
Herring	129,751
Sardines	72,345

There are two major classes of fish in Table 1 (small pelagic fish and larger carnivorous fish). The small pelagic fish consists of menhaden, herring, and sardines; and the larger carnivorous fish consists of pollock, salmon, cod, flatfish, and hakes. In the US, only menhaden (a small pelagic fish with high oil content) is

harvested solely for making fishmeal and fish oil. In other parts of the world, harvesting fish in an unsustainable manner for meal and oil production has had detrimental effects on marine ecosystems.[16, 17] Some marine scientists feel that current world fishmeal and fish oil production may not be sustainable; however, most are in agreement that utilizing fish wastes for producing protein meals and oils is more desirable than discarding these protein and oil rich raw materials.

Creating a Sustainable Environment for both the Fishmeal and Fish Oil Industry and the Growing Aquaculture Industry

One way to reduce the strain on the fishmeal and fish oil industry is to increase the use of plant protein sources in aqua and agriculture feeds. There are many factors to consider when choosing a plant based feed ingredient. One of the biggest factors is the amount of antinutrients found in many plant based byproducts, such as toxic amino acids, saponins, cyanogenic glycosides, tannins, phytic acid, gossypol, oxalates, goitrogens, lectins, protease inhibitors, chlorogenic acid, and amylase inhibitors.[18,19] Despite these antinutrients, there is no shortage of plant based feed ingredients, therefore, agricultural engineers are working on new plant byproduct processing methods to remove the bulk amount of these antinutrients from plant byproducts. In addition to reducing the amount of antinutrients in plant based feeds, it is also important to identify other

nutritionally valuable compounds that can be supplemented into plant based feeds to increase the nutritional quality of the feed. This can be accomplished by identifying individual compounds or groups of compounds in fish byproduct fractions that result in enhanced growth responses in feeding trials. It is probable that nutritional scientists will develop plant based feeds either supplemented with a certain fraction of fish byproduct or with commercially available compounds of interest based on detailed analyses of the compounds in fish byproducts. There have been extensive studies on developing plant based feeds for use in catfish aquaculture that have proven to be as successful as fishmeal feeds.[20-23] Also, much progress has been made with farmed rainbow trout, salmon, shrimp and other species using plant based feed ingredients.[24-26] All of these studies suggest that fishmeal, in many situations, can be replaced or reduced with cheaper plant protein meals and oils.

It is known that plant and marine byproducts differ from each other in terms of protein content, free amino acid compositions, lipid compositions, and other soluble nitrogen containing components.[27] Many studies have focused on the compositions of amino acids, proteins, and lipids, but the identification of small molecular weight soluble nitrogen containing components in marine resources has recently become of increased interest. One recent study showed that the inclusion of stickwater in plant based

aquaculture feeds stimulates growth in salmon.[28] In that study protein content, and lipid content of the diets were similar, but addition of the small water soluble components in the stickwater stimulated growth in the salmon. Stickwater is a very complex mix of proteins and small molecular weight compounds making it difficult to identify the biologically active components. In addition to identifying important individual compounds in stickwater, it is important to consider that much of the nutritional value of stickwater may be due to a group of compounds rather than any individual chemical compound. These groups of compounds may work together to optimize a series of metabolic pathways leading to increased growth responses that cannot be triggered by any individual metabolite.

The Renewed Interest in the Composition of Stickwater

Stickwater is the aqueous fraction of fishmeal and typically represents 20-40% of the total protein of the processed fish byproduct. Stickwater contains high concentrations of "water soluble" proteins, minerals, and small molecular weight organic molecules, which represent the majority of the stickwater solids. In many large fishmeal plants, stickwater is hydrolyzed with enzymes so it can be more easily concentrated in evaporators, and then the concentrated stickwater (30-40% solids) is often sprayed on the wet protein press cake and dried. Stickwater is usually not dried to a powder because of the difficulty and expense in the final drying stage. Some smaller

fishmeal plants discard the stickwater, such as commercial plants that make fishmeal and oil from processing byproduct but do not have evaporators for concentration (i.e. at-sea processors). Typically if concentrated stickwater is added to fishmeal, its value is that of the price of fishmeal minus concentration costs.

It is distinctly possible that value added components can be isolated from stickwater and incorporated into plant based fish feeds resulting in an improved plant based feed. By developing enriched feed ingredients from stickwater, fishmeal plants will have new ingredients for the aquaculture industry. Figure 1 shows the movement of fish and fish byproducts from a commercial fish processor to the world market. Fishmeal plants that are equipped with the fractionation and drying system necessary to produce stickwater fractions will be able to utilize their stickwater and possibly produce new feed ingredients instead of discarding the stickwater. Finding new uses for stickwater or stickwater fractions are of interest to several industries including the aquaculture and fishmeal industries. These new proposed uses for stickwater are shown in Figure 1 as “enriched feed ingredients.”

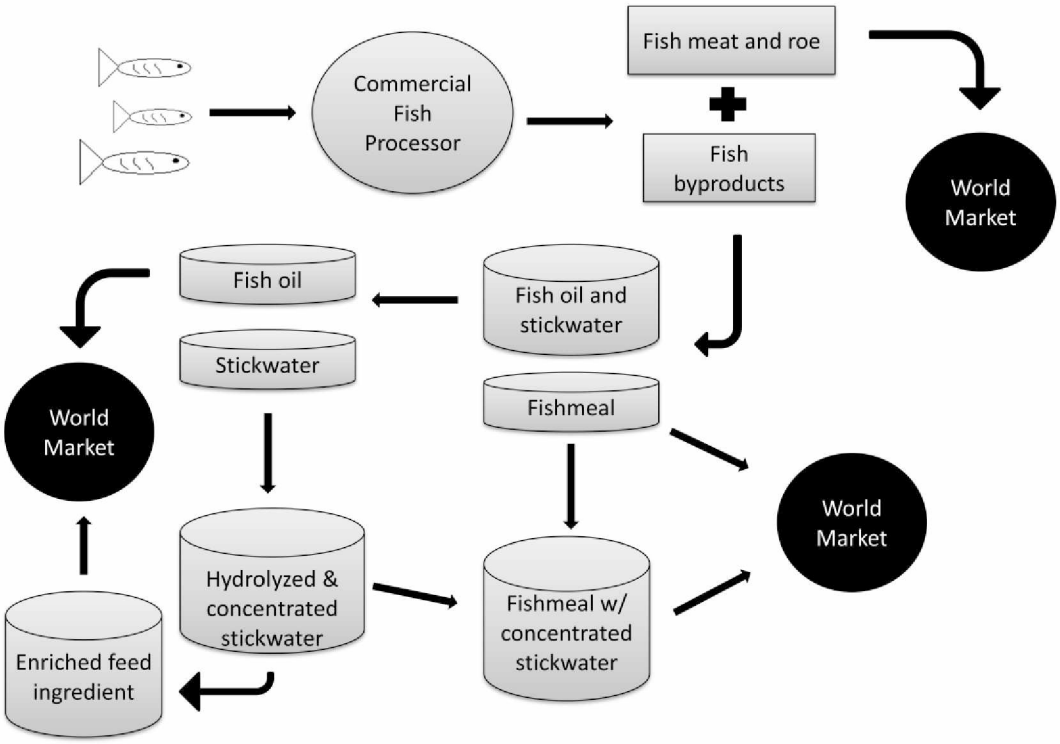

Figure 1: Diagram showing the movement of fish and fish byproduct from commercial processors to the world market.

In the past, many studies identified that it was the amino acid composition and the water soluble protein concentrations that made stickwater a valuable component to aquaculture and agriculture feeds.[29-31] Recent research on metabolic pathways have allowed researchers to identify potential nitrogen containing compounds (i.e. free amino acids, quaternary ammonium compounds, nucleotides, nucleosides, and organic acids) that play important roles in growth and metabolism. Many of these nitrogen containing compounds that are found in fish would be expected to be found primarily in the aqueous stickwater fraction. These nitrogen containing

compounds found in stickwater are potentially of economic importance to the fishmeal industry.

Analysis of tissues from marine organisms can be used to identify potential growth and metabolic stimulants in fish and other marine organisms that are not present in plant based protein meals. One study has found that many marine fish contain high concentrations of creatine, taurine, inosine, hypoxanthine, histidine, and alanine which led the authors to conclude that some or all of these compounds might play important roles in the growth of marine fish.[32] Other studies on potential nitrogen containing compounds of interest found in fish byproduct suggest that in addition to the compounds mentioned above, anserine and carnitine may play important roles in fish nutrition.[27,28] The identification of these potentially important metabolites and other compounds of interest has led to the need for the development of rapid instrumental methods capable of the simultaneous, qualitative, and quantitative analysis of many different classes of low molecular weight nitrogen containing compounds in complex fish byproduct samples.

Hydrophilic Interaction Liquid Interface Chromatography (HILIC) for the Separation of Low Molecular Weight Nitrogen Containing Compounds

In order to identify and quantify the low molecular weight nitrogen containing compounds, a suitable method capable of separating a variety of nitrogen containing compounds must be developed. Most of the target compounds are not very volatile and are in low concentration. Thus, it would be very difficult to perform the needed separation using gas chromatography (GC) without derivatization. Therefore, high performance liquid chromatography (HPLC) was chosen for the separation. Reverse phase HPLC works well for the separation of ionizable compounds and biological extracts, however very polar molecules such as amino acids are not well retained and must be derivatized prior to separation. Many derivatization techniques have been developed for the analysis of amino acids via reverse phase separations.[33-38] Since many of the nitrogen compounds in stickwater come from varying classes of compounds, a single derivatization technique would not work well for the analysis.

In 1990, Dr. Andrew Alpert developed an HPLC technique called HILIC.[39] HILIC separations utilize hydrophilic interactions between the target analytes and the stationary phase in order to separate the different analytes. The HILIC mode of separation has proven to be successful at separating

various biomolecules without derivitization, whereas most reverse phase techniques used to separate these types of molecules require derivatization of the target analytes.[40-43] The fact that the HILIC mode of separation is capable of separating many highly polar low molecular weight molecules (without derivatization) makes it the separation technique of choice for this project.

The Fishmeal and Fish Oil Industry in Alaska and this Project

In 2009, the Alaska Department of Fish and Game (ADFG) estimated that commercial processors harvested 1.5 billion dollars (exvessel value) of seafood from Alaskan fisheries, which had a wholesale value of over 3 billion dollars.[44] The majority of the fish caught in these fisheries are groundfish (i.e. pollock, cod, etc) followed by salmon, herring, shellfish, and halibut, respectively. The estimated weight averages of commercially harvested fish in Alaska over a five year period from 2005-2009 are shown in Table 2.[44]

Table 2: Average annual commercial landings of fish in Alaska (2005-2009).

Type of Fish	Average Annual Landings (in metric tons)
Groundfish	1,950,000
Salmon	412,000
Herring	37,285
Halibut	23,451

The State of Alaska Department of Economic Development Office of Fisheries Development has conservatively estimated that the 10 year average from 2000-2009 of commercially processed fishmeal and fish oil are 49,000 metric tons of fishmeal and 9,500 metric tons of fish oil, which is worth over

100 million dollars annually.[45] Much of the byproduct from small salmon processors and offshore catcher-processors is not used; however, more byproduct would be processed if it was of greater value. In order to utilize these byproducts more effectively, research is being conducted to look at different byproduct fractions and how the isolation of different fractions can lead to more nutritionally enriched products.

The focus of this project is to develop a robust, non-derivatizing HILIC-HPLC method for the separation, identification, and quantification of low molecular weight nitrogen containing compounds in fish byproduct samples such as stickwater fractions. It is hypothesized that the majority of the low molecular weight nitrogen containing compounds will partition primarily to the aqueous stickwater phase during fishmeal processing. Thus, enriched fractions can be prepared from stickwater for use as feed ingredients and supplements as illustrated in Figure 1. The fish byproduct samples used for this method development and initial application are from red salmon (*Oncorhynus nerka*).

Chapter 2: Experimental Methods

Sampling

Fresh Alaskan red salmon (*Oncorhynchus nerka*) samples were collected from a commercial processing plant in Kodiak, AK. The samples were then processed at the Fishery Industrial Technology Center (FITC) in Kodiak, AK during August of 2010. Fishmeal and stickwater samples were obtained during normal operations from the Kodiak Fishmeal Company in Kodiak, AK. Two stickwater samples were taken: the first sample (pre-treated) was taken immediately after the oil was removed, and before the hydrolytic enzyme was added to the stickwater. This dilute stickwater sample is 6-8% solids. The second sample (post-treated) was taken after the addition of commercial protease (alcalase) and concentration in evaporators of the stickwater to 30-40% solids. Three separate 15 liter samples of both pre-treated and post-treated stickwater samples were collected, poured into trays, and frozen over night at -30°C. After freezing, the samples were freeze dried (Virtis Virtual 52ES) and stored at -30°C. Fishmeal samples obtained from the Kodiak Fishmeal Company were predominantly from salmon byproduct and had concentrated stickwater added back to the press cake before final drying. The fishmeal samples were stored frozen until transport to Fairbanks, AK.

Whole fish (red salmon) samples were processed by grinding three fresh red salmon through 3/8 inch holes in the grinder plate. The ground samples were placed in separate trays overnight at -30˚C and freeze dried. All freeze dried samples were vacuum packaged and kept at -30°C until the samples were transported to Fairbanks, AK for analysis. Upon arrival in Fairbanks, the samples were stored in a -80˚C freezer until analysis.

Chemicals and Reagents

All chemical and chromatographic reagents were of HPLC grade. Acetonitrile and ammonium formate were purchased from Acros Organics (Pittsburgh, PA). Formic acid was purchased from Fischer Scientific (Pittsburgh, PA). Amino acids, amines, organic acids, nucleic bases, nucleosides, and nucleotides were purchased from Acros Organics (Pittsburgh, PA), Sigma-Aldrich (St. Louis, MO), Tokyo Chemical Industry (Tokyo, Japan), Fluka (Buchs, Switzerland), Agilent Technologies (Waldbronn, Germany), Promega Corp (Madison, WI), and CalbioChem (Darmstadt, Germany). Type I ASTM (American Society for the Testing of Materials) water was prepared using a Barnstead (Dubuque, IA) water filtration unit (model #D11931).

Chromatographic Instrumentation and Conditions

The chromatographic analyses were carried out using an Agilent Technologies (Santa Clara, CA) 1100 series HPLC equipped with a degasser, gradient pump, autosampler, and diode array detector coupled to a Sedere (Alfortville Cedex, France) Sedex 85 low temperature evaporative light scattering detector (LT-ELSD). The separation column stationary phase was a Phenomenex (Torrance, CA) Luna HILIC column (150 mm x 4.6 mm, 3 µm). The mobile phase used in the method development was a mixture of acetonitrile and an aqueous solution of ammonium formate: 90% (by volume) acetonitrile and 10% (by volume) 1mM ammonium formate brought to a pH of 3 with concentrated formic acid. Isocratic conditions were optimized at a flow rate of 1 mL/minute with an injection volume of 5 µL.

Preparation of Samples

The fishmeal and freeze dried fish byproduct samples (pre-treated stickwater, post-treated stickwater, and whole fish samples) were diluted to volume in ASTM Type I water using the dilutions shown in Table 3. The prepared diluted samples were dissolved and vortexed for two minutes. The samples were then centrifuged in an IEC (Newtown, CT) Clinical Centrifuge at approximately 1,000 G for five minutes. After centrifuging the sample, a three mL aliquot of the aqueous phase was transferred to a Milli-Q 3000 MW centrifugal filtration filter and centrifuged in a Sorvall (Newtown, CT) RC 5B

Plus Centrifuge at 9,500 G for 25 minutes. A two mL aliquot of the diffusate was transferred to an HPLC vial for analysis.

Table 3: Fish byproduct dilutions used for sample preparation.

Sample	Weight of sample	Dilution volume
Whole fish	0.6 g	25 mL
Pre-treated stickwater	0.3 g	25 mL
Post-treated stickwater	0.3 g	25 mL
Fishmeal	0.6 g	25 mL

Preparation of Spiked Samples

Individual 1500 ppm (mg/L) standards were prepared for amino acids, organic acids, nucleic bases, and nucleosides. A five mL aliquot of the 1500 ppm standard was added to a dry sample and diluted to volume as shown in Table 3. The prepared diluted spiked samples were dissolved and vortexed for two minutes. The samples were then centrifuged in an IEC (Newtown, CT) Clinical Centrifuge at approximately 1,000 G for five minutes. After centrifuging the sample a three mL aliquot of the aqueous phase was transferred to a Milli-Q 3000 MW centrifugal filtration filter and centrifuged in a Sorvall (Newtown, CT) RC 5B Plus Centrifuge at 9,500 G for 25 minutes. A two mL aliquot of the diffusate was transferred to an HPLC vial for analysis.

Preparation of Standards for Calibration

Amino acids, amines, organic acids, nucleic bases, and nucleotides were prepared as a mixed standard at a concentration of 1000 ppm in ASTM Type I water. The 1000 ppm mixed standard sample was then diluted to 100, 200, 250, 300, 400, and 500 ppm concentrations. A three mL aliquot of the prepared mixed standards were transferred to Milli-Q 3000 MW centrifugal filtration filter tubes and centrifuged in a Sorvall (Newtown, CT) RC 5B Plus super speed centrifuge at 9,500 g's for 25 minutes. A two mL aliquot of the diffusate was transferred from each filter tube to an HPLC vial for analysis.

Chapter 3: Results

Optimization of HPLC and ELSD Operational Parameters

The HPLC operational parameters were optimized by evaluating the chromatograms produced from triplicate replications of pre-treated stickwater samples under the following conditions:

- Mobile phase composition: acetonitrile /water

  The compositions evaluated were 90%/10%, 80%/20%, and 70%/30%

- Aqueous mobile phase composition: salt concentration and pH

  Two concentrations of ammonium formate were evaluated 1mM and 10 mM.

  The two ammonium formate solutions were evaluated at pH 3 and pH 6 using formic acid to adjust the pH.

- HPLC flow rate:

  Flow rates were evaluated from 0.5 mL/min to 1.5 mL/min in increments of 0.25 mL/min.

- Column temperature:

  The column temperature setting was evaluated from 20-40°C in increments of 5°C

Various combinations of these parameters were run over the course of several days. The injection volume and dry weight of the sample used were not evaluated at this time. Instead, a set injection volume of 5 µL was used with the maximum dry weight of sample per volume of solvent (25 mg/mL suggested by Millipore for use in their centrifugal filtration tubes) was used for all of the optimization runs. The chromatograms produced were evaluated and the final optimized settings determined from the optimization runs were: 90%/10% acetonitrile to water (mobile phase composition), 1mM ammonium formate brought to a pH of 3 with formic acid (aqueous phase), 1mL/min (flow rate), and 25˚C (column temperature). Using the optimized HPLC settings above, the ELSD settings were optimized as follows:

- Gain (signal intensity):Intensity = 2^n where n=gain

  The gain was evaluated from settings of 1-9.
- Detector Temperature:

  The detector temperature was evaluated from 25-45˚C in increments of five degrees.

The detector settings were evaluated and a gain of three and a temperature of 30˚C were selected for the method. After developing the method, there were changes made to the dry weight sample dilutions of the pre-treated and post-treated stickwater samples in order to optimize separation of the components in these samples. The dry weight loadings of

the stickwater samples were rediluted, since these samples are the most concentrated samples and required further dilution to get optimal separation. The injection volume of the four types of samples (whole fish, pre-treated stickwater, post-treated, and fishmeal) was kept at 5 µL. Figures 2-5 show a typical chromatogram for pre and post-treated stickwater, whole fish, and fishmeal).

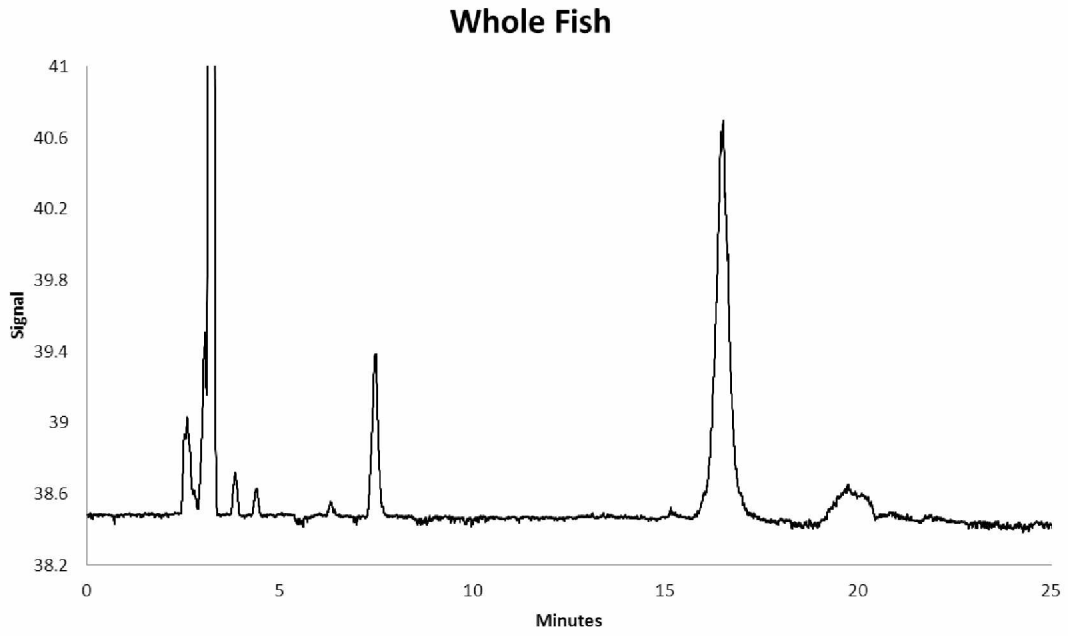

Figure 2: Chromatogram of freeze-dried whole fish produced from the optimized HPLC method.

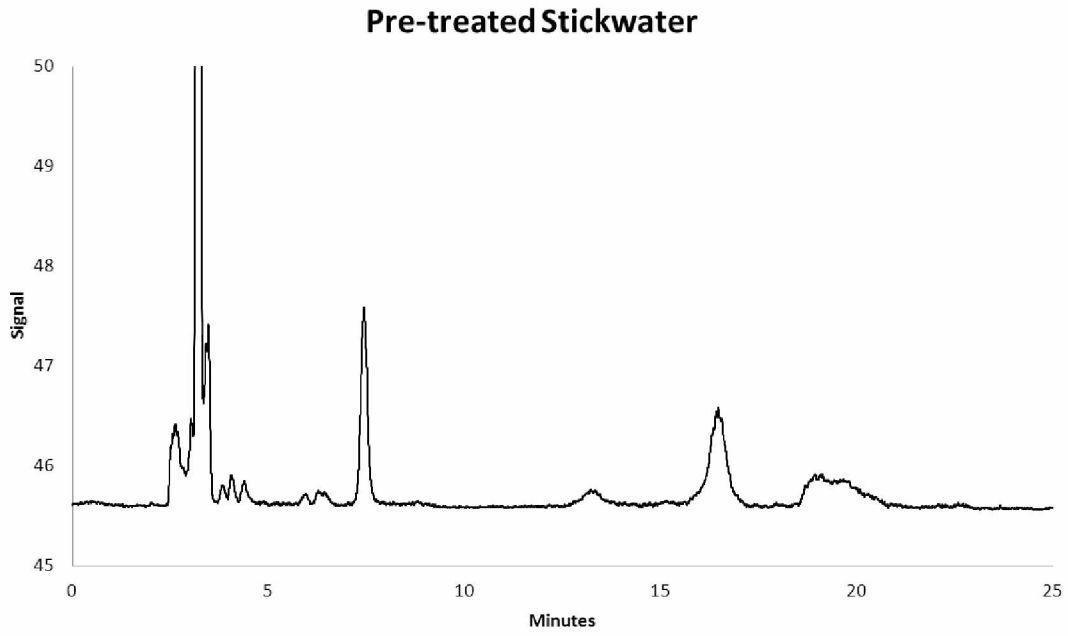

Figure 3: Chromatogram of freeze-dried pre-treated stickwater produced from the optimized HPLC method.

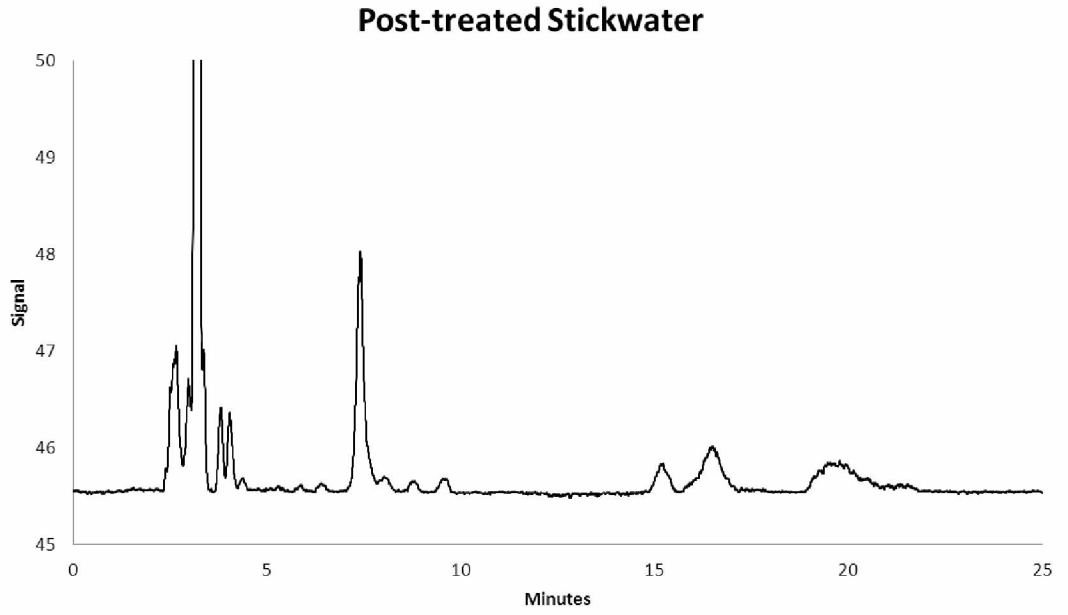

Figure 4: Chromatogram of freeze-dried post-treated stickwater produced from the optimized HPLC method.

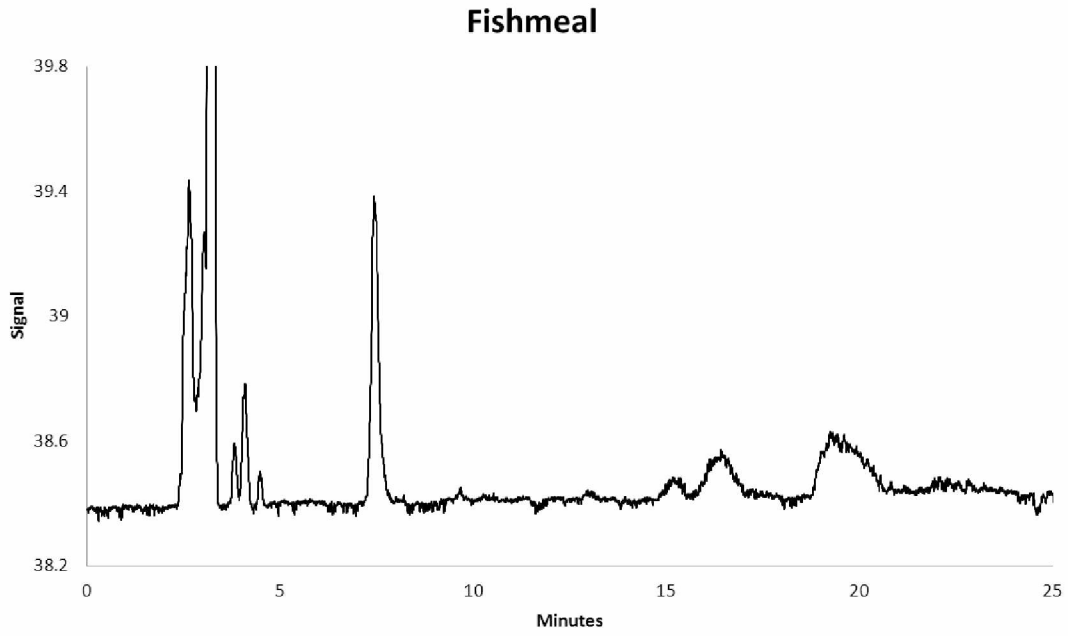

Figure 5: Chromatogram of fishmeal produced from the optimized HPLC method.

The chromatograms in Figures 2-5 show good peak separation and a short run time of 25 minutes. The chromatograms all appear to contain the same main components; however there appear to be some peaks in the post-treated stickwater (hydrolyzed) sample that are not observed in the other chromatograms. These small peaks only seen in the post-treated sample are most likely free amino acids, since the alcalase treatment enzymatically hydrolyzes protein.

Identification of Analytes in the Samples

A set of sample spiking experiments was utilized to identify the analyte peaks observed in Figures 2-5. The analyte spikes were approximately 300 ppm for each of the prepared spiked samples. A list of the target analytes and their respective retention times are shown in Table 4. The identified peaks in all four sample chromatograms are shown in Figure 6. The chloride based salt identification was made based on the identification of two spiked peaks in samples prepared using a hydrochloride form of another compound (i.e. trimethylamine hydrochloride and tyrosine hydrochloride). There are three peaks of interest yet to be identified. The first peak not identified is eluted following creatinine and is observed in three of the chromatograms (pre-treated stickwater, post-treated stickwater, and fishmeal). Another peak not identified was observed in the pre-treated stickwater samples at approximately 13 minutes, and the

Table 4: Retention times recorded from the analyte spiking experiments.

Analyte	Time (Minutes)
trimethylamine	2.8
hypoxanthine	3.8
creatinine	4.1
taurine	7.5
leucine	7.6
isoleucine	8
tryptophan	8.8
methionine	8.8
proline	8.9
valine	9.6
tyrosine	11
hydroxyproline	12.8
carnitine	12.9
threonine	14.6
alanine	15
creatine	16.7
glutamic acid	19.8
serine	20
lactic acid	20.5
glutamine	21
aspartic acid	23.8

final peak not identified was observed in both the post-treated stickwater samples and the fishmeal samples at approximately 15 minutes.

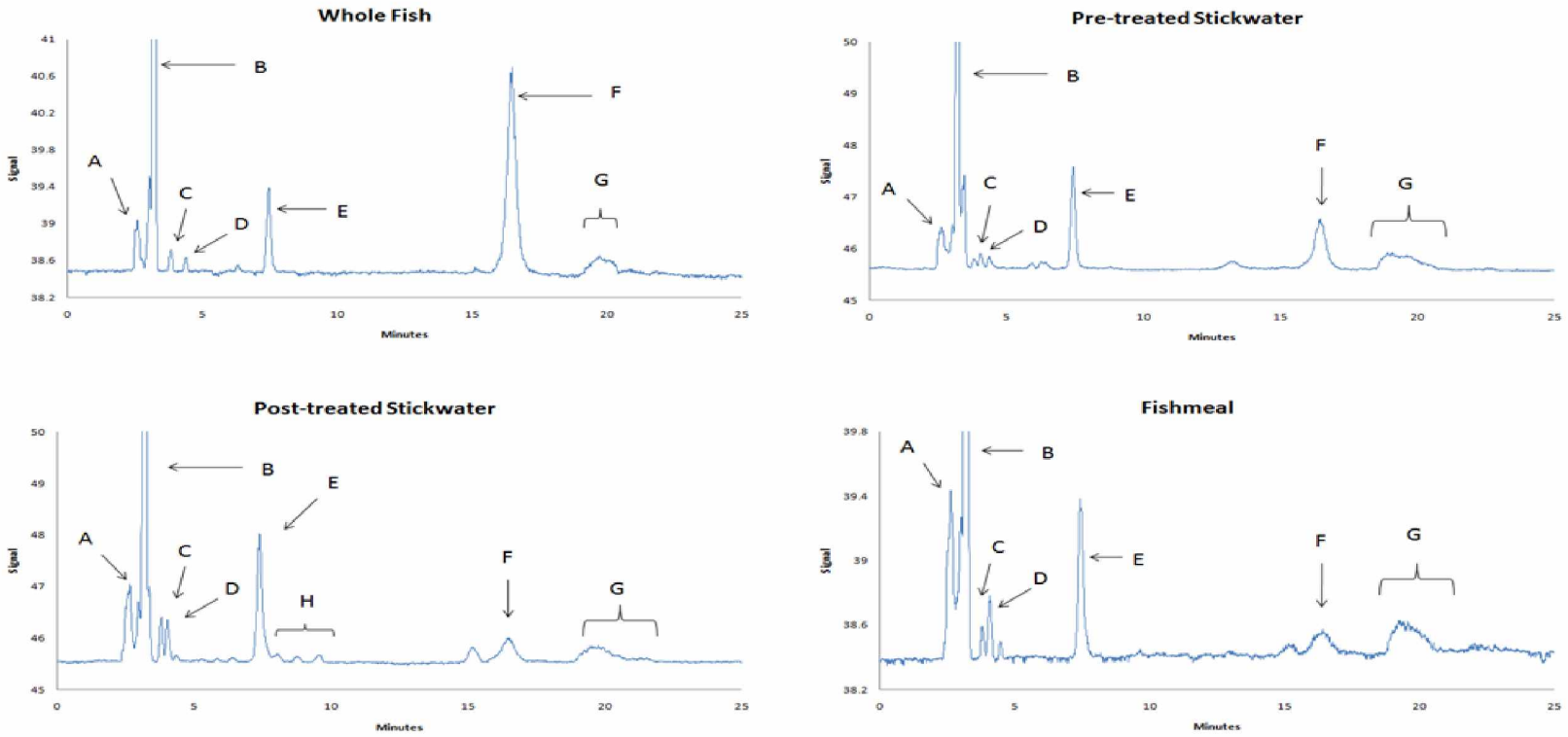

Figure Legend

A= Trimethylamine	D= Creatinine	G= Glutamic acid and Glutamine
B= Chloride based salt	E= Taurine	H= Isoleucine, Leucine, and Methionine
C= Hypoxanthine	F= Creatine	

Figure 6: Sample chromatograms from the four red salmon byproduct fractions with the identified compounds labeled.

Method Percent Recovery

L-carnitine was chosen as the analyte for the percent recovery study. The retention time for L-carnitine in the salmon byproduct samples was 12.9 minutes. All four of the samples have baseline detector measurements around this time. The fishmeal byproduct sample was chosen for the percent recovery study. The biggest losses attributed to this method are due to the water soluble analytes not passing through the filter membrane. The fishmeal samples leave the most residue on the membrane filters which can prevent water soluble analytes from crossing the membrane. Therefore, the fishmeal samples would have the biggest percent loss. Figure 7 is a chromatogram of fishmeal with a L-carnitine spike.

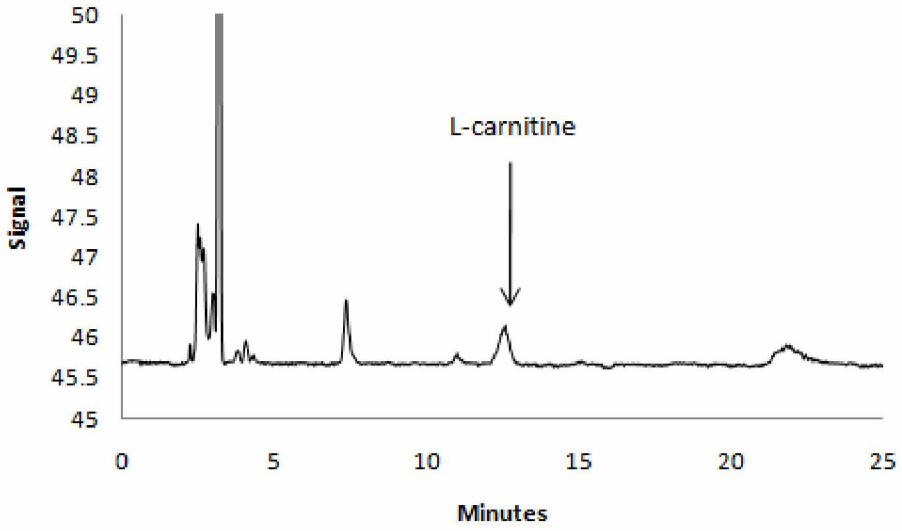

Figure 7: Chromatogram of a fishmeal sample with a L-carnitine spike.

A set of six fishmeal samples were spiked with 234.7 ppm of L-carnitine and duplicate analyses were run for each sample. The results are shown in Table 5. The samples are labeled 1-6 and the duplicate analyses are identified as A and B.

Table 5: Results from the percent recovery experiment.

Sample ID	Fishmeal (g) in 25 mL H_2O	Retention time (min)	Signal Area	ppm recovered	% recovery
1A	0.5895	12.5	11.0	230.6	98.3
1B	0.5895	12.7	11.0	230.6	98.3
2A	0.5883	12.7	11.2	233.1	99.3
2B	0.5883	12.5	10.6	225.5	96.1
3A	0.5821	12.6	10.3	221.6	94.4
3B	0.5821	12.7	10.5	224.2	95.5
4A	0.5565	12.5	10.9	229.3	97.7
4B	0.5565	12.7	11.5	236.8	100.9
5A	0.5624	12.6	11.7	239.3	102.0
5B	0.5624	12.6	12.0	243.0	103.5
6A	0.5690	12.8	9.7	213.8	91.1
6B	0.5690	12.6	11.2	233.1	99.3

The results of the percent recovery experiment had an average percent recovery of 98% ± 3%. Overall the method works well with very limited analyte loss. Analysis of the duplicate replications shows that the method has good reproducibility.

Quantification Results:

The major identified peaks with the exception of trimethylamine and chloride based salt peaks were all quantified. All of the quantified analytes had a signal to noise ratio of at least 8:1. A set of nine sub-samples were prepared from each salmon sample sent from Kodiak, AK (whole fish, pre-treated stickwater, post-treated stickwater, and fishmeal). Each of the nine samples was analyzed with duplicate replications. The results of the quantification study are shown in Table 6. The standard deviations calculated for each of the analytes in each of the samples indicate that the method is reproducible and gives reliable results.

Table 6: Quantification results from the fish byproduct samples. Averages and standard deviations are in grams of analyte per kilogram of dry sample.

Compound	**Whole Fish**	**Pre-treated Stickwater**	**Post-treated Stickwater**	**Fishmeal**
	Avg ± Stddev (g/kg)	Avg ± Stddev (g/kg)	Avg ± Stddev (g/kg)	Avg ± Stddev (g/kg)
Hypoxanthine	1.9 ± 0.3	7 ± 1	6 ± 1	1.5 ± 0.1
Creatinine	1.7 ± 0.3	3.3 ± 0.7	6.4 ± 0.7	2.5 ± 0.1
Taurine	6.7 ± 0.8	29 ± 4	25 ± 3	8.6 ± 0.3
Creatine	21 ± 3	32 ± 7	19 ± 3	5.5 ± 0.7

The trimethylamine peak was not quantified due to the poor calibration curve produced from the standard calibration. The poor calibration curve of this compound is likely due to the properties of the evaporative light scattering detector and the volatile nature of trimethylamine. The volatile nature of trimethylamine led to inconsistencies

in the amount of trimethylamine in the standards. For the rest of the quantified analytes, the calibration curves provided a good fit to power functions. The power function curve fits were expected, since ELSD do not produce linear calibration curves.[46] A typical calibration curve is shown for each of the quantified analytes and for L-carnitine (quantified during the percent recovery experiment) in Figures 8-12. The calibration curves produced were reproducible day to day with little fluctuation. A sample mixed standard chromatogram is shown in Figure 13. In the Appendix, supplemental data tables are shown from the quantification runs.

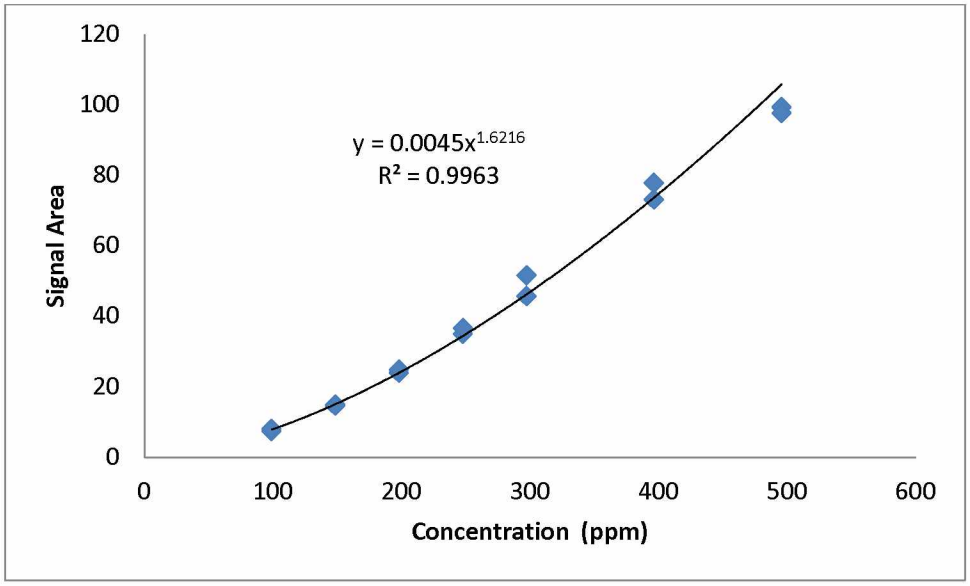

Figure 8: Sample standard calibration curve used to quantify hypoxanthine.

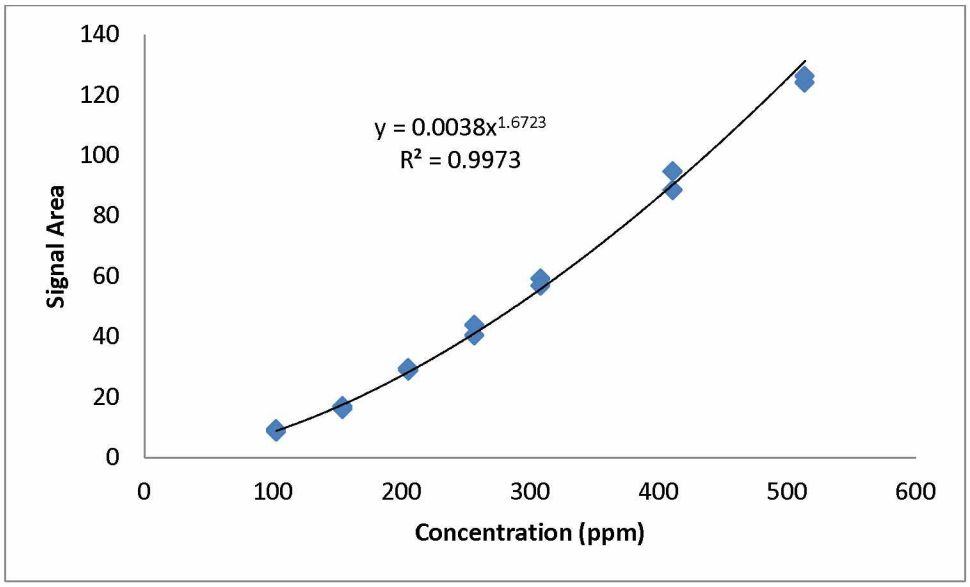

Figure 9: Sample standard calibration curve used to quantify creatinine.

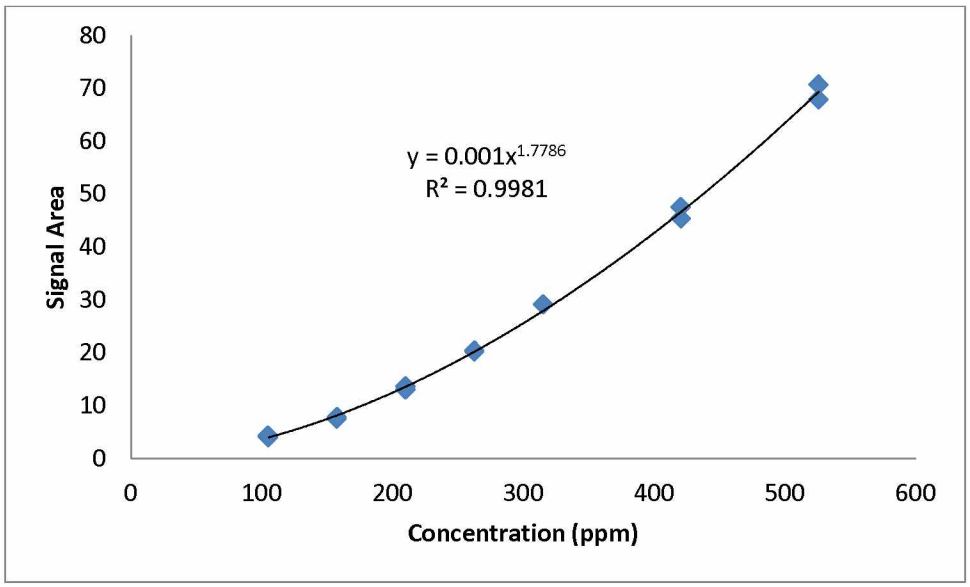

Figure 10: Sample standard calibration curve used to quantify taurine.

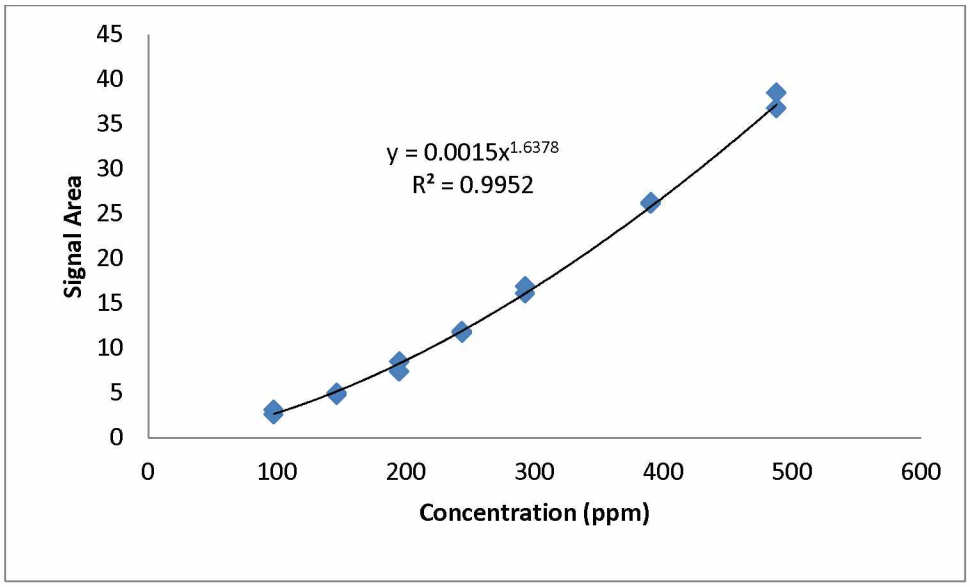

Figure 11: Sample standard calibration curve used to quantify creatine.

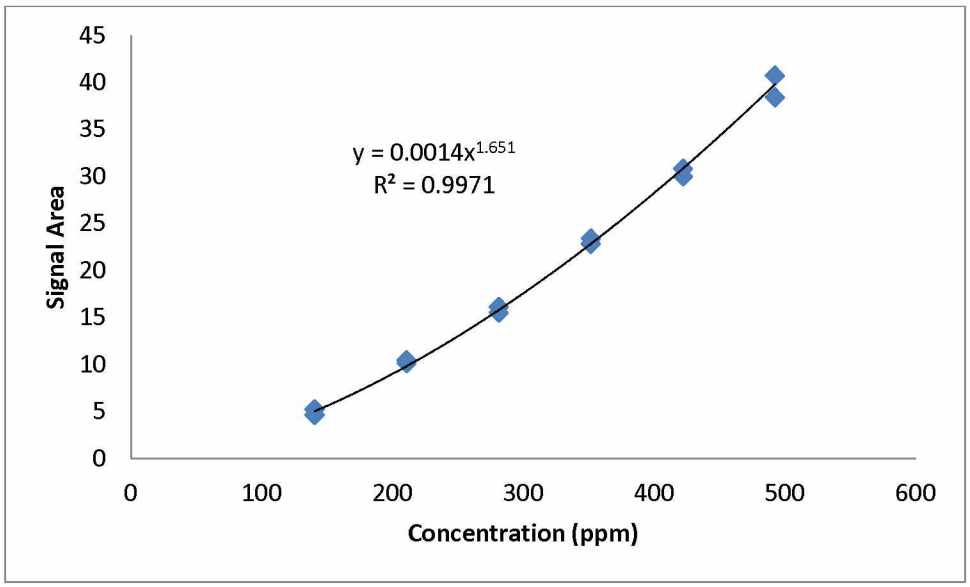

Figure 12: Sample standard calibration curve used to quantify L-carnitine (percent recovery).

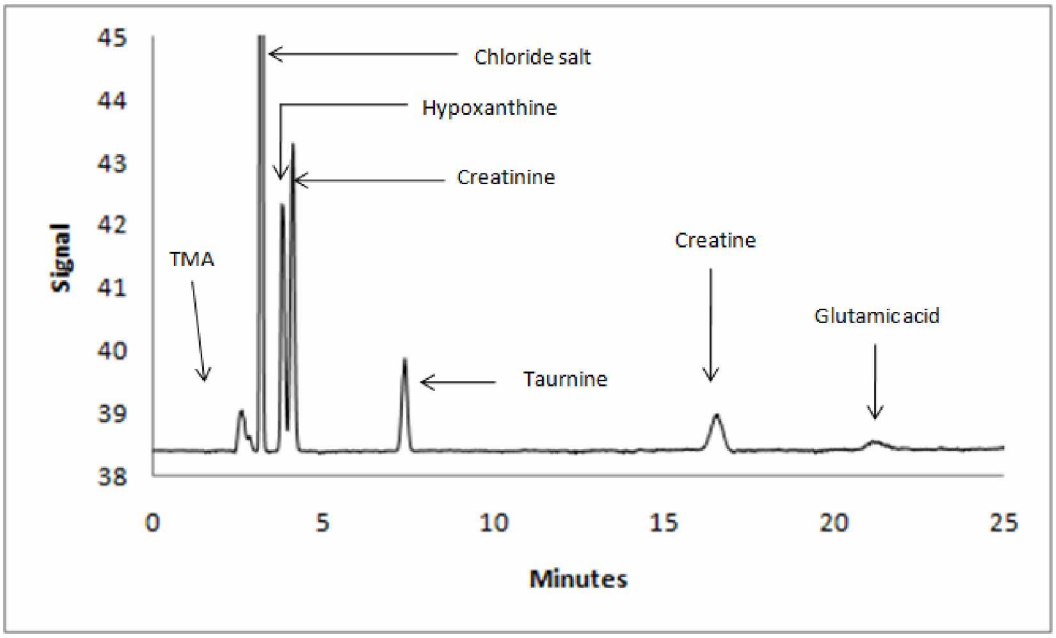

Figure 13: Typical chromatogram of a mixed standard.

Chapter 4: Discussion

Optimization of the HPLC and ELSD Operational Parameters

The optimization of the run settings for this HILIC-HPLC/ELSD method resulted in three key findings:

1. The more organic phase used the more likely the method will work well for a low temperature evaporative light scattering detector.
2. There is an important relationship between the column temperature and the ELSD temperature.
3. It is important to select the appropriate aqueous phase conditions for optimization of the ELSD.

It was the identification of these three points that allowed for the method to result in good resolution and reproducibility for the HPLC/ELSD method.

One of the most important aspects to developing this method was the role of the organic mobile phase. When developing a method for an ELSD, it was important to take into consideration the type of organic solvent and amount of organic solvent used for the mobile phase. The greater the volatility of the organic phase and the more organic phase used leads to an increased signal to noise ratio. HILIC methods use a greater percentage of organic phase as compared to the aqueous phase which will typically minimize the noise on an ELSD. On the other hand, many reverse phase

techniques use a larger aqueous phase composition which isn't as well evaporated at low temperatures as a volatile organic phase and leads to more noise in the chromatograms using an ELSD.

In addition, it is important to consider the column temperature and detector temperature together. The detector temperature is dependent upon the composition of the mobile phase, and the column temperature has a similar dependence upon the detector temperature. By setting the column temperature lower than the detector temperature, the peak shape of the chromatograms improved resulting in an increase in the signal to noise ratio.

The composition of the aqueous phase played an important role in the development of this method. The salt concentration of the mobile phase should be minimized to reduce the noise in the baseline. The noise is directly affected by salt composition when an ELSD is used, since the salt is continuously being eluted and detected by the detector. The pH of the mobile phase is very important to separating the analytes in the solution. By reducing the pH, the analytes in solution become ionized which increases the hydrophilic interactions between the column and the analytes. The analytes eluted in the first five minutes were not well resolved when an aqueous phase with a pH of 6 was used; however, the aqueous phase with a pH of 3 ionized these analytes which increased their polarity making them more hydrophilic and ultimately resulted in greater separation.

Identification of the Analytes

Using sample spiking methods, the majority of the compounds were identified as labeled in Figure 6. The most prevalent compounds identified in the different fish byproduct fractions were trimethylamine, chloride salt, hypoxanthine, creatinine, taurine, creatine, glutamic acid, and glutamate. The post-treated stickwater samples contain three amino acids (isoleucine, leucine, and methionine) that are not observed at these concentrations in any of the other samples. This was expected since the post treatment involved hydrolysis of protein which would increase the concentrations of free amino acids. The identified compounds are in good agreement with the compounds identified and quantified in red salmon tissue as reported by Carr et al.[31] The identification of all of these different types of analytes in a single HPLC method demonstrates the robust nature of the method. There are four peaks of interest yet to be identified. The first peak not identified is eluted following creatinine and is observed in three of the chromatograms (pre-treated stickwater, post-treated stickwater, and fishmeal). The second peak not identified is eluted around six minutes and is observed in the whole fish, pre-treated stickwater, and post-treated stickwater. The other two major peaks not identified were observed in the pre-treated stickwater samples at approximately 13 minutes, and the other peak was observed in both the

stickwater samples and the fishmeal samples at approximately 15 minutes. None of these peaks have been identified.

Percent Recovery and Quantification Results

The percent recovery of the preparative sample clean-up was 98% ± 3%. These results indicate that this method has little analyte loss associated with the method. The chromatograms and the standard deviations from the quantification results show that the method is highly reproducible with little deviations in the replicates. The identified compounds above the limit of quantification (>LOQ) were quantified with the exception of trimethylamine, chloride salt, and the overlapping peaks of glutamic acid, glutamate, and aspartic acid. Trimethylamine was not quantified due to its volatile nature and poor ELSD calibration results. The chloride salt is not a nitrogen containing compound and was thus excluded from the quantification results. There is interest in quantifying the glutamic acid, glutamate, and aspartic acid overlapping peaks; however, the method developed is not capable of separating these peaks.

The tabulated results in Table 6 indicate that there is significant partitioning of these low molecular weight nitrogen containing compounds to the stickwater fraction. The concentrations of the analytes in the fishmeal samples are between 25-45% of the concentration of the analytes in the pre-treated and post-treated stickwater samples. These results indicate that

nearly all of the low-molecular weight nitrogen containing compounds quantified completely partition to the stickwater fraction, since the concentrations of these compounds in the fishmeal samples can be accounted for by the incorporation of stickwater into the fishmeal prior to drying. The pre and post-treated stickwater have similar quantifed profiles; however there were amino acid peaks qualitatively identified in the post-treated stickwater that are not observed in the pre-treated stickwater. These amino acids are due to the use of the alcalase treatment which enzymatically hydrolyzes protein and frees amino acids from proteins. Overall, these results indicate this method can be used to better understand the complex array of small nitrogen containing organic compounds in stickwater, which will ultimately support better utilization of fish byproducts.

Chapter 5: Conclusions

Conclusions from the Method Development and Quantification Experiments

The HILIC method developed allowed for the quick separation, identification, and quantification of low molecular weight compounds in fish byproduct fractions. In addition, the developed preparative method clean-up had a very high percent recovery (98% ± 3%). Unlike reverse phase separations, this method does not require derivatization. The HILIC method has a much higher percent recovery, since there was no derivatization required of the target analytes in the samples. Figures 2-5 show adequate separation of the different low molecular weight components in the fish byproduct fractions. The separation of the target analyte peaks in the figures allowed for the identification of several compounds using sample spiking and retention time matching. The identified compounds in the samples are in good agreement with the compounds identified in red salmon tissue.[32]

The quantified results indicate that there is a great deal of partitioning of these low molecular weight nitrogen containing components to the stickwater fraction during fishmeal processing. The most concentrated of these compounds were hypoxanthine, creatinine, taurine, and creatine. The findings suggest that feed ingredients consisting of fraction(s) enriched with hypoxanthine, creatinine, taurine, and creatine could be prepared from

stickwater. There is also the possibility of further purification of the compounds. By developing enriched feed ingredients from stickwater, stickwater may be utilized by small processors that are currently disposing of their stickwater. Potential markets for the enriched or partially purified stickwater fractions include feed ingredients and supplements for aquaculture/agriculture, and as food ingredients and supplements in human diets. Other industrial uses may also be developed.

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Appendix:

Table A1 is supplemental data collected from the whole fish quantification runs. Table A2 is supplemental data collected from the pre-treated stickwater quantification runs. Table A3 is supplemental data collected from the post-treated stickwater quantification runs. Table A4 is supplemental data collected from the fishmeal quantification runs. The sample ids are named as follows: the first number represents the sample set it came from (i.e. all sample types sent from Kodiak, Ak were sent in triplicate form). The letter following the number indicates the sub sampling (i.e three samples were taken from each of the three samples from each set. For each of the quantified analytes there is one column showing the signal area and a column showing the quantified concentration in mg of analyte per kg of dry weight.

Table A1: Supplemental data collected from the whole fish quantification runs.

Sample id	weight	Hyp (sig)	Hyp (mg/g)	Crn (sig)	Crn (mg/g)	Tau (sig)	Tau (mg/g)	Cre (sig)	Cre (mg/g)
1A	0.6205	2.00	2.07	1.30	1.51	9.20	6.72	44.00	23.43
1A	0.6205	1.70	1.87	1.10	1.37	9.80	6.97	50.90	25.42
1B	0.6000	1.60	1.87	1.50	1.70	9.40	7.04	41.00	23.29
1B	0.6000	1.80	2.01	1.40	1.63	9.40	7.04	39.80	22.91
1C	0.6024	1.20	1.56	1.00	1.33	8.80	6.75	42.60	23.70
1C	0.6024	1.60	1.86	1.00	1.33	8.60	6.66	41.60	23.39
2A	0.6183	1.50	1.74	2.10	2.01	10.50	7.27	41.30	22.70
2A	0.6183	2.40	2.31	2.60	2.28	12.10	7.88	37.50	21.50
2B	0.5945	2.30	2.35	2.20	2.15	11.90	8.12	37.10	22.23
2B	0.5945	2.20	2.28	1.90	1.97	10.20	7.44	29.50	19.56
2C	0.5907	2.80	2.66	1.80	1.92	9.70	7.28	24.50	17.74
2C	0.5907	2.50	2.48	1.60	1.79	10.00	7.40	21.30	16.41
3A	0.6119	1.60	1.83	1.20	1.46	6.90	5.79	24.80	17.24
3A	0.6119	1.30	1.62	1.30	1.53	6.80	5.74	27.10	18.12
3B	0.6003	1.60	1.87	1.00	1.33	6.50	5.71	27.60	18.66
3B	0.6003	1.60	1.87	1.10	1.41	6.10	5.51	28.00	18.81
3C	0.5774	1.40	1.79	1.10	1.47	6.30	5.83	33.40	21.58
3C	0.5774	1.00	1.46	1.10	1.47	6.60	5.98	37.00	22.86

Hyp= hypoxanthine, Crn= creatinine, Tau= taurine, Cre= creatine
Sig = signal area, mg/g = mg of target analyte per g dry weight of

Table A2: Supplemental data collected from the pre-treated stickwater quantification

Sample id	weight	Hyp (sig)	Hyp (mg/g)	Crn (sig)	Crn (mg/g)	Tau (sig)	Tau (mg/g)	Cre (sig)	Cre (mg/g)
1A	0.3213	2.70	6.87	0.80	2.38	40.80	31.30	13.20	24.49
1A	0.3213	2.70	6.87	1.30	3.14	40.60	31.22	13.20	24.49
1B	0.3061	2.60	7.06	0.70	2.32	37.10	31.22	11.70	23.70
1B	0.3061	2.40	6.75	0.80	2.50	34.80	30.16	12.10	24.25
1C	0.3009	1.60	5.48	2.00	4.29	22.90	24.48	14.90	28.38
1C	0.3009	1.30	4.87	2.10	4.41	22.70	24.37	15.30	28.89
2A	0.3062	1.20	4.58	1.60	3.71	19.50	22.06	11.70	23.70
2A	0.3062	0.90	3.90	1.40	3.44	19.00	21.76	12.30	24.51
2B	0.3221	3.30	7.67	1.10	2.85	42.40	31.88	18.40	30.57
2B	0.3221	3.60	8.05	1.30	3.14	42.80	32.04	16.90	28.86
2C	0.3192	3.60	8.13	1.10	2.88	47.20	34.08	20.90	33.61
2C	0.3192	3.30	7.74	0.90	2.57	42.20	32.09	21.70	34.47
3A	0.3306	3.10	7.22	1.80	3.68	43.50	31.49	24.40	36.02
3A	0.3306	3.20	7.35	1.50	3.32	43.30	31.41	24.20	35.82
3B	0.3201	3.20	7.59	1.50	3.42	40.40	31.25	26.80	39.63
3B	0.3201	3.20	7.59	1.50	3.42	40.80	31.42	29.30	42.09
3C	0.3016	1.60	5.46	2.10	4.40	22.70	24.31	29.20	44.57
3C	0.3016	1.70	5.65	2.20	4.52	22.60	24.25	24.80	39.92

Hyp= hypoxanthine, Crn= creatinine, Tau= taurine, Cre= creatine
Sig = signal area, mg/g = mg of target analyte per g dry weight of

Table A3: Supplemental data collected from the post-treated stickwater quantification

Sample id	weight	Hyp (sig)	Hyp (mg/g)	Crn (sig)	Crn (mg/g)	Tau (sig)	Tau (mg/g)	Cre (sig)	Cre (mg/g)
1A	0.3098	3.70	5.06	5.30	6.13	19.50	20.84	7.90	15.11
1A	0.3098	3.40	4.81	4.60	5.63	22.00	22.30	7.00	14.03
1B	0.3374	4.20	5.03	5.40	5.69	24.80	21.90	14.60	20.18
1B	0.3374	4.40	5.17	5.20	5.56	25.00	22.00	12.50	18.35
1C	0.3010	6.90	7.65	6.90	7.38	32.10	28.39	16.70	24.56
1C	0.3010	6.60	7.45	7.00	7.45	33.00	28.83	13.70	21.76
2A	0.3180	3.90	5.10	5.20	5.90	23.90	22.76	11.70	18.70
2A	0.3180	4.40	5.49	5.70	6.23	22.50	22.00	9.90	16.89
2B	0.3298	8.40	7.89	7.20	6.91	36.80	27.98	11.90	18.22
2B	0.3298	8.10	7.71	6.90	6.74	33.80	26.67	11.20	17.56
2C	0.3211	4.40	5.44	5.70	6.17	23.10	22.12	11.50	18.33
2C	0.3211	3.80	4.97	5.10	5.78	24.10	22.65	12.10	18.91
3A	0.3029	6.80	7.54	6.20	6.88	31.20	27.76	14.30	22.20
3A	0.3029	6.10	7.05	5.30	6.27	32.20	28.26	12.30	20.25
3B	0.3301	7.60	7.41	7.60	7.13	37.10	28.08	14.20	20.28
3B	0.3301	8.20	7.76	8.10	7.41	39.70	29.17	14.50	20.54
3C	0.3336	4.50	5.31	6.00	6.13	28.30	23.86	15.80	21.42
3C	0.3336	4.60	5.38	5.50	5.82	27.60	23.53	14.30	20.15

Hyp= hypoxanthine, Crn= creatinine, Tau= taurine, Cre= creatine
Sig = signal area, mg/g = mg of target analyte per g dry weight of

Table A4: Supplemental data collected from the fishmeal quantification runs.

Sample id	weight	Hyp (sig)	Hyp (mg/g)	Crn (sig)	Crn (mg/g)	Tau (sig)	Tau (mg/g)	Cre (sig)	Cre (mg/g)
1A	0.608	1.70	1.63	3.30	2.38	12.30	8.37	2.60	4.01
1A	0.608	1.50	1.51	3.10	2.30	12.20	8.33	3.60	4.85
1B	0.5495	1.20	1.45	2.80	2.39	10.90	8.66	3.20	5.01
1B	0.5495	1.40	1.60	2.80	2.39	10.80	8.62	4.70	6.29
1C	0.5697	1.40	1.54	2.80	2.31	10.70	8.27	3.00	4.65
1C	0.5697	1.10	1.32	2.80	2.31	11.10	8.44	4.50	5.91
2A	0.57	1.50	1.61	2.80	2.30	11.70	8.68	4.10	5.59
2A	0.57	1.20	1.40	3.30	2.54	11.60	8.64	4.50	5.91
2B	0.5934	1.20	1.34	3.30	2.44	12.20	8.54	4.70	5.82
2B	0.5934	1.40	1.48	3.10	2.35	11.50	8.26	4.80	5.89
2C	0.5967	1.50	1.54	3.70	2.60	13.20	8.86	5.50	6.35
2C	0.5967	1.30	1.40	3.70	2.60	12.60	8.64	4.60	5.72
3A	0.6048	1.40	1.45	3.30	2.39	12.00	8.30	3.00	4.38
3A	0.6048	1.80	1.70	4.10	2.72	13.40	8.82	4.70	5.71
3B	0.5856	1.30	1.43	3.60	2.60	13.30	9.07	5.00	6.12
3B	0.5856	1.20	1.36	3.20	2.43	13.50	9.15	5.50	6.47
3C	0.594	1.40	1.48	3.40	2.48	12.60	8.68	4.50	5.67
3C	0.594	1.50	1.54	4.00	2.73	13.30	8.94	4.10	5.36

Hyp= hypoxanthine, Crn= creatinine, Tau= taurine, Cre= creatine
Sig = signal area, mg/g = mg of target analyte per g dry weight of